



RESEARCH ARTICLE



## *Tuber borchii* Shapes the Ectomycorrhizosphere Microbial Communities of *Corylus avellana*

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### ABSTRACT

In this study, eight-month-old ectomycorrhizae of *Tuber borchii* with *Corylus avellana* were synthesized to explore the influence of *T. borchii* colonization on the soil properties and the microbial communities associated with *C. avellana* during the early symbiotic stage. The results showed that the bacterial richness and diversity in the ectomycorrhizae were significantly higher than those in the control roots, whereas the fungal diversity was not changed in response to *T. borchii* colonization. *Tuber* was the dominant taxon (82.97%) in ectomycorrhizae. Some pathogenic fungi, including *Ilyonectria* and *Podospora*, and other competitive mycorrhizal fungi, such as *Hymenochaete*, had significantly lower abundance in the *T. borchii* inoculation treatment. It was found that the ectomycorrhizae of *C. avellana* contained some more abundant bacterial genera (e.g., *Rhizobium*, *Pedomicrobium*, *Ilumatobacter*, *Streptomyces*, and *Geobacillus*) and fungal genera (e.g., *Trechispora* and *Humicola*) than the control roots. The properties of rhizosphere soils were also changed by *T. borchii* colonization, like available nitrogen, available phosphorus and exchangeable magnesium, which indicated a feedback effect of mycorrhizal synthesis on soil properties. Overall, this work highlighted the interactions between the symbionts and the microbes present in the host, which shed light on our understanding of the ecological functions of *T. borchii* and facilitate its commercial cultivation.

### ARTICLE HISTORY

Received 25 December 2018  
Revised 22 April 2019  
Accepted 28 April 2019

### KEYWORDS

*Tuber borchii*;  
ectomycorrhizae; microbial  
communities; *Corylus  
avellana*; soil

## 1. Introduction

Worldwide, there are at least 180–230 species of truffles, which are a type of ectomycorrhizal fungi with high economic value [1]. *Tuber borchii* Vittad., which is a complex truffle species that has a strong smell during its mature stage, is widely distributed throughout Europe [2,3]. Since its first cultivation, *T. borchii* has become popular and it is now commonly used in dishes in northeast Italy; therefore, it has a broad prospect in the edible fungus market [4,5]. *T. borchii* has a relatively wide adaptability to host plants, which can form symbiotic relationships with hazel, oak, poplar, chestnut, alder, linden, and coniferous species, such as cedar and pine [2]. *T. borchii* spores can also produce well-formed ectomycorrhizae on *Carya illinoensis* (pecan) and *Quercus franchetii* seedlings [6,7]. In addition to its wide host range, *T. borchii* also has a relatively wide adaptability to soils and climates when compared

with species such as *T. magnatum* and *T. melanosporum* [6–8]. Truffles generally need calcareous soil of pH 7–8, but *T. borchii* is also able to withstand slightly acidic soil [9]. Moreover, molecular biological studies have shown that *T. borchii* have saprobic behaviors during mycelium reproduction under pure culture conditions as well as in the fruiting bodies [10,11]. *C. avellana*, which are well known for their capacity to form ectomycorrhizae, are commonly used as mycorrhizal hosts for *T. melanosporum* [12,13]. In this study, we utilized the spores of *T. borchii* to infect *C. avellana* to observe the formation of ectomycorrhizae and then explore its microbial diversity.

Mycorrhizae are an important bridge between root systems and soil, which can contribute to the improvement of plant nutrition [14,15]. The development of this symbiont can be influenced by microbes in the mycorrhizosphere [16–19]. There are also abundant and diverse bacteria and fungi

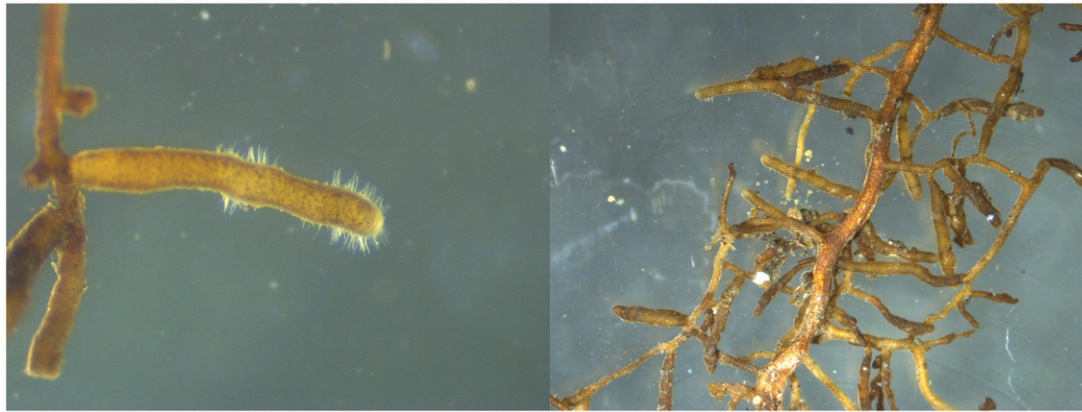
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Supplemental data for this article can be accessed [here](#).

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**Figure 1.** Root tips of *C. avellana* in association with *T. borchii*.

present in the ectomycorrhizae and fruiting bodies of truffles. It has been reported that these microbial communities are affected by season, region, and truffle species [20–22]. Moreover, different microbes have been isolated from the hyphae of ectomycorrhizal fungi and fruiting bodies [23].  $\alpha$ -Proteobacteria were found to be the major communities related to the ascoma of truffles, independently of the degree of maturation [18,24]. In terms of *T. borchii*, researchers have assumed various roles for bacterial communities related to ascoma during the growth and maturation of ascocarps [25,26]. Nevertheless, the effects of truffle ectomycorrhizal symbiosis on the soil properties, the other microbes in soils, as well as the interaction between microbes in host plants and symbionts are not clear [22]. Endophytes are associated with the metabolic processes of host plants and are important to the growth and fitness of the host [27,28]. Understanding the effects of truffle ectomycorrhizal symbiosis on microbes in the host will facilitate cultivation of *T. borchii* and help reveal the mechanism of the formation of truffle ectomycorrhizae in the natural environment. In this study, we analyzed the rhizosphere soil properties of the host as well as the microbial communities in ectomycorrhizae using next-generation sequencing technology with the goal of exploring how *T. borchii* shapes the microbial communities in the ectomycorrhizae of *C. avellana*.

## 2. Materials and methods

### 2.1. Cultivation of *C. avellana* seedlings and *T. borchii* inoculation

*C. avellana* seeds were cultivated in greenhouses according to previously described methods [22,29]. Briefly, *C. avellana* seeds were surface-sterilized. The nursery substrate, which was composed of vermiculite, perlite, and water (at a volume ratio of 1:1:1), was then sterilized in an autoclave for 2 hours at 121 °C, after which the surface-sterilized seeds were sown in prepared nursery substrate. After two to three months, the seedlings were transplanted into a

container with 1 L sterilized cultivation substrate, which was composed of peat, organic soil, vermiculite, and water at a volume ratio of 1:1:1:1.5 (pH = 7.5). Inoculation was conducted at the time of transplanting. Briefly, surface-sterilized ascocarps of *T. borchii* were blended to spore powder [22], after which about two grams of the spore powder was inoculated into the cultivation substrate. Take the *C. avellana* seedlings that were not inoculated with *T. borchii* spores as controls. The number of inoculated and uninoculated seedlings was both five. All of the seedlings were maintained in the greenhouse under the same conditions. During the cultivation period, plants were watered every day, and fertilizers were not applied [29].

### 2.2. Sampling strategy and soil analyses

After 8 months of cultivation, *C. avellana* seedlings were removed from the substrate and the root tips and rhizosphere soils were gathered. The chemical properties of rhizosphere soils were then measured according to the method previously described by Li et al. [30]. The effects of soil chemical properties on microbial communities were evaluated using canonical correspondence analysis (CCA) [31]. *C. avellana* roots were rinsed with sterile water prior to morphological and molecular analyses of ectomycorrhizae. Mycorrhization was successfully detected in seedlings inoculated with truffle spores (Figure 1). The root tips were then removed and placed in 2-ml centrifuge tubes using sterilized tweezers. The soil and root samples were divided into two treatments, including *T. borchii* inoculation treatment and control treatment (seedlings without *T. borchii* inoculation). Soil samples had three replicates and root samples had five replicates. Root tips of *C. avellana* mycorrhized with *T. borchii* were designated as “B” and the ectomycorrhizosphere soil was designated as “B.S.” Root tips from control plants that were not inoculated with *T. borchii* were designated

**Table 1.** Physical and chemical properties of *C. avellana* rhizosphere and ectomycorrhizosphere soils.

Sample	pH	OM (g/kg)	TN (g/kg)	TP (g/kg)	TK (g/kg)	AN (mg/kg)	AP (mg/kg)	AK (mg/kg)	ECa cmol (1/2Ca <sup>2+</sup> )/kg	EMg cmol (1/2Mg <sup>2+</sup> )/kg
Cr.S.	8.37 ± 0.01*	90.00 ± 1.61	3.20 ± 0.02	1.15 ± 0.03	25.13 ± 0.26*	212.00 ± 1.41	21.60 ± 0.67	112.00 ± 1.63	67.03	1.23
B.S.	8.11 ± 0.01	89.20 ± 0.79	3.29 ± 0.01*	1.22 ± 0.03	23.72 ± 0.14	231.33 ± 6.02*	24.23 ± 0.26*	143.67 ± 0.47*	66.37 ± 0.9	4.70 ± 0.14*

OM: organic matter; TN: total nitrogen; TP: total phosphorus; TK: total potassium; AN: effective nitrogen; AP: available phosphorus; AK: available potassium; ECa: exchangeable calcium; EMg: exchangeable magnesium; Cr.S.: rhizosphere soil; B.S.: ectomycorrhizosphere soil. Each value is the mean of three replicates (±SD). “\*” indicates significant difference between samples ( $p < 0.05$ ).

as “Cr” and the surrounding soils were designated as “Cr.S.”

### 2.3. DNA extraction and MiSeq sequencing

Before DNA extraction, the ectomycorrhizae or control roots of *C. avellana* were washed with distilled sterile water and surface-disinfected with 75% alcohol [30]. The effectiveness of the surface sterilization was determined by the growth of microorganisms. The hexadecyltrimethyl ammonium bromide (CTAB) method was used to extract the genomic DNA of endophytes and tissues [22]. The extracted genomic DNA was detected by 1% agarose gel electrophoresis. The universal primers 515 F-806R and ITS1F-ITS2 were used to amplify the 16S V4 and ITS1 region of all of the samples. High-fidelity DNA polymerase (NEB Q5) was employed to ensure the efficiency and accuracy of PCR amplification [32]. The results of the PCR amplification were checked by 2% agarose gel electrophoresis, after which the target fragments were cut and recovered using an Axygen gel recycling kit. The PCR products of each sample were then quantified using a Quant-iT Pico Green dsDNA Assay Kit with a microplate reader (Bio Tek, FL × 800), mixed and detected by 2% agarose gel electrophoresis. An Illumina TruSeq Nano DNA LT Library Prep Kit was used for library construction. The final segment selection and purification of the library was made through 2% agarose gel electrophoresis. Paired-end sequencing (2 × 300 bp) was then conducted on the Illumina MiSeq platform using the V3 MiSeq Reagent Kit over 600 cycles.

### 2.4. Sequence data analysis

After the double end sequencing of DNA, the original data were saved in FASTQ format. The QIIME software was then used to identify and eliminate the query sequence and USEARCH (v5.2.236) was employed to check and remove the chimera sequences to obtain high-quality sequences [33]. The number of high-quality sequences of each sample and the length distribution of the high-quality sequences in the whole sample were calculated using the R software.

High-quality sequences with ≥97% nucleotide similarity were clustered into operational taxonomic

units (OTUs) with Mothur and UCLUST (<https://unite.ut.ee/>) [34]. The taxonomic classifications of bacterial and fungal OTUs were assigned using the RDP 3 classifier and the SILVA (Release119 <http://www.arb-silva.de>) and UNITE (Release 6.0 <http://unite.ut.ee/index.php>) databases, respectively. Shared OTU abundances were visualized using a Venn diagram. Rarefaction curves were calculated in QIIME. Alpha-diversity of microbial communities was reflected by four indices (Chao1, Coverage, Shannon, and Simpson). The β-diversity and the relationships between samples were analyzed using principal component analysis (PCA) in the R environment (v.2.13.1) [35]. Linear discriminant analysis effect size (LEfSe) was used to detect the microbial taxa that had different relative abundances between the treatment and the control groups at the genus level using the Galaxy online analysis platform. GraPhlAn, a visualization tool [36], was used to build up a hierarchical tree of the sample population at each taxonomic level. Different colors were used to distinguish between the taxonomic units and the abundance of the taxonomic units was reflected by the node size. A heatmap was employed to show the difference in abundance of the 50 most abundant genera between the microbial community samples using the R software. Metabolic functions of bacterial communities were predicted using the PICRUSt software based on the KEGG database. The functional gene categories and the abundance of the coding genes in each sample are shown in the bar charts.

Statistical differences were assessed using one-way analysis of variance (ANOVA) in the SPSS 19.0 software package. Data of this study are presented as the means ± standard deviation (SD) for each treatment group. The least significant difference (LSD) was determined for pair-wise comparison of the differences between treatment groups following ANOVA. A  $P < 0.05$  was considered to indicate significance.

## 3. Results

### 3.1. Analysis of rhizosphere and ectomycorrhizosphere soil properties

Some soil physicochemical characteristics around *C. avellana* roots with or without *T. borchii*



**Table 2.** Richness and diversity of bacterial and fungal communities associated with *C. avellana* roots with or without *T. borchii* symbionts.

Index	Bacterial		Fungal	
	B	Cr	B	Cr
Observed species	1642 ± 139.55**	1176.6 ± 185.15	209.4 ± 79.33	181.6 ± 62.58
Shannon	5.01 ± 0.79*	2.94 ± 1.21	1.8 ± 0.87	2.146 ± 0.45
Simpson	0.75 ± 0.09*	0.46 ± 0.19	0.45 ± 0.16	0.66 ± 0.12
Chao1	1339.13 ± 157.07*	823.82 ± 175.43	172.82 ± 86.12	137.15 ± 56.05
ACE	1339.14 ± 156.57**	830.40 ± 166.98	173.96 ± 85.72	142.60 ± 59.96

B, ectomycorrhizae from *C. avellana* mycorrhized with *T. borchii*. Cr, roots from cultivated *C. avellana* without *T. borchii* partner. Each value is the mean of 5 replicates (± SD). "\*" indicates significant differences between samples ( $p < 0.05$ ).

mycorrhization are presented in Table 1. The pH and total potassium of the B.S. was significantly lower than that of the Cr.S. ( $p < 0.05$ ). The organic matter, total phosphorus, and exchangeable calcium of the B.S. were similar to those in the Cr.S., with no significant differences. The content of total nitrogen, available nitrogen, available phosphorus, available potassium, and exchangeable magnesium was significantly higher in the B.S. than the Cr.S. ( $p < 0.05$ ).

### 3.2. Bacterial alpha diversity in ectomycorrhizae and roots

A total of 60, 627–82, 264 high-quality sequences were obtained from each sample after quality control procedures (Figure S1a). In total, 2354 bacterial OTUs of each sample were clustered into 38 phyla, 100 classes, and 718 genera. The numbers of observed species in treatment B were significantly higher than in the control treatment ( $p < 0.05$ ) (Table 2). The Chao1 and ACE indices, which represent the richness of community, showed that the bacteria were significantly more abundant in treatment B ( $p < 0.05$ ). The Simpson and Shannon indices, representing community diversity, indicated that the bacterial diversity of ectomycorrhizae was significantly higher than that of the control group ( $p < 0.05$ ).

### 3.3. Fungal alpha diversity in ectomycorrhizae and roots

About 39, 802–93, 557 high-quality sequences were obtained in each sample (Figure S1b). In total, 72–223 fungal OTUs were present in each sample and they were assigned to 11 phyla, 29 classes, and 195 genera. There were no significant differences in any of the indices between treatments (Table 2); therefore, the fungal community richness and diversity of treatment B were similar to those of the control group.

### 3.4. GraPhlAn visualization of annotated phylogeny and taxonomy of microbes

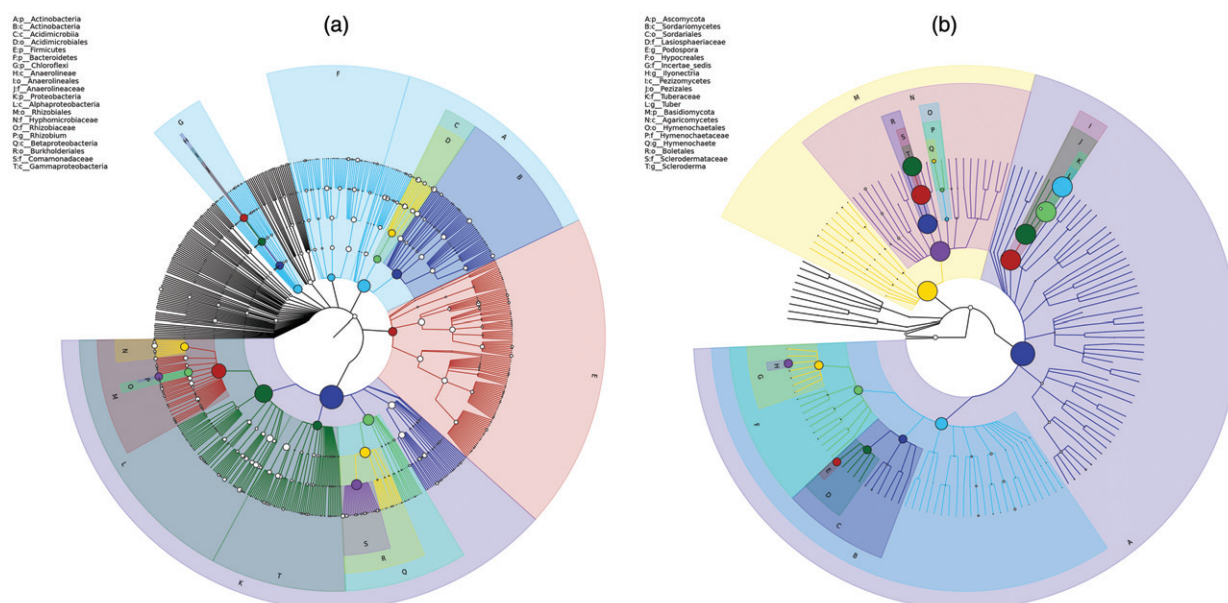
GraPhlAn trees (Figure 2) were used to visualize the circular taxonomic and phylogenetic structures of bacterial and fungal taxa from the phylum to the genus level. Differences in the microbial communities in *C. avellana* roots with or without *T. borchii* partner at all taxonomic levels are clearly illustrated in the GraPhlAn tree. The relative abundances of the top 20 taxa were clearly illustrated in the GraPhlAn tree.

The bacterial distributions in *C. avellana* roots indicated that Proteobacteria clustered to the largest branch, followed by Firmicutes, Acidobacteria, Bacteroidetes, and Chloroflexi (Figure 2(a)). The dominant classes within Proteobacteria were  $\alpha$ -proteobacteria,  $\beta$ -proteobacteria, and  $\gamma$ -proteobacteria. About 20% of the tree was occupied by Firmicutes which was mainly composed of Bacilli at the class level. Additionally, 20% of the tree was composed of Acidobacteria, including the classes Actinobacteria and Acidimicrobiia. Acidimicrobiales was the dominant order of Acidimicrobiia, while Anaerolineae was the dominant class in Chloroflexi.

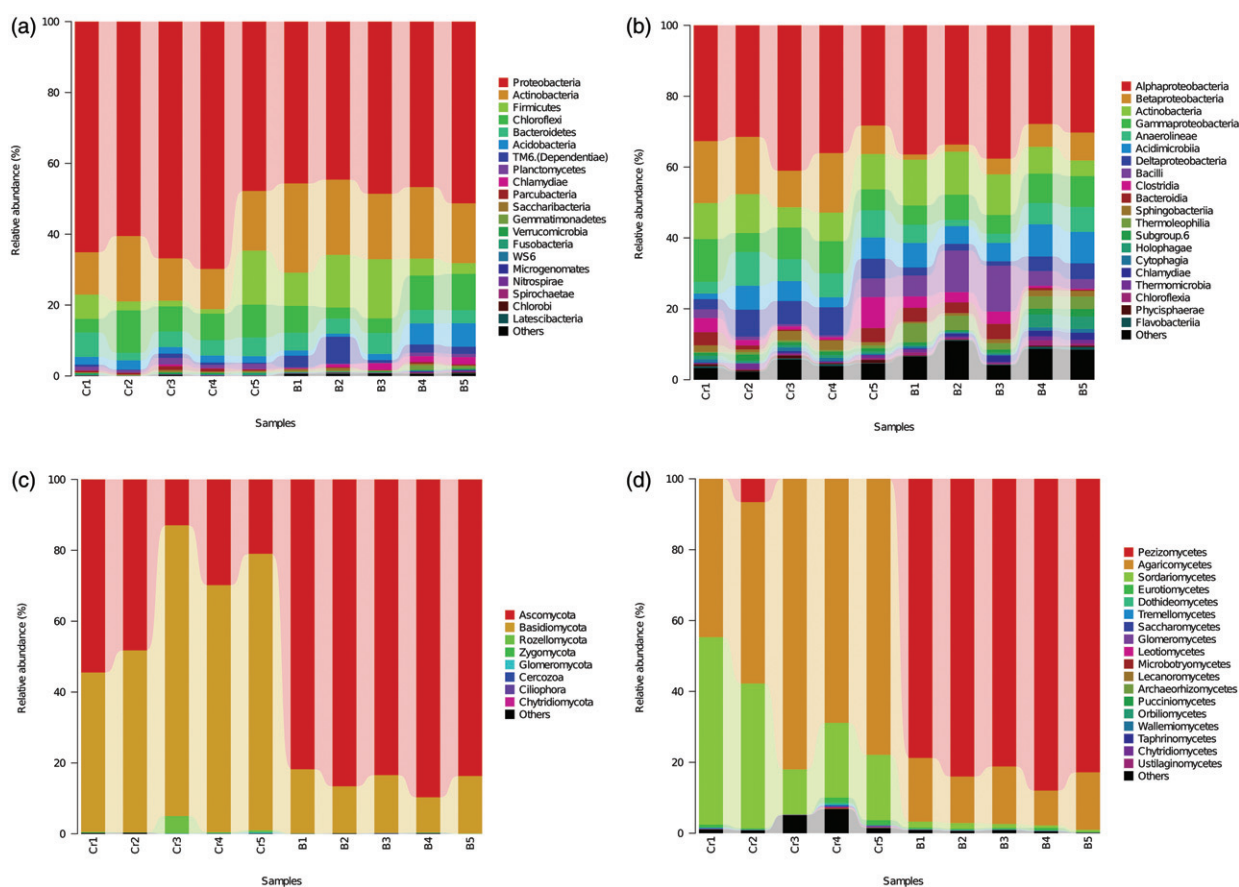
*C. avellana* roots harboured two major fungal phyla, Ascomycota and Basidiomycota (Figure 2(b)). The dominant classes within Ascomycota were Sordariomycetes and Pezizomycetes. When compared with Sordariomycetes, Pezizomycetes, which mainly contains members of the order Pezizales, was significantly more abundant. The dominant family within Pezizales was Tuberaceae, of which *Tuber* was the most abundant genus and was only found in samples of treatment B.

### 3.5. Taxonomic analyses of bacterial communities

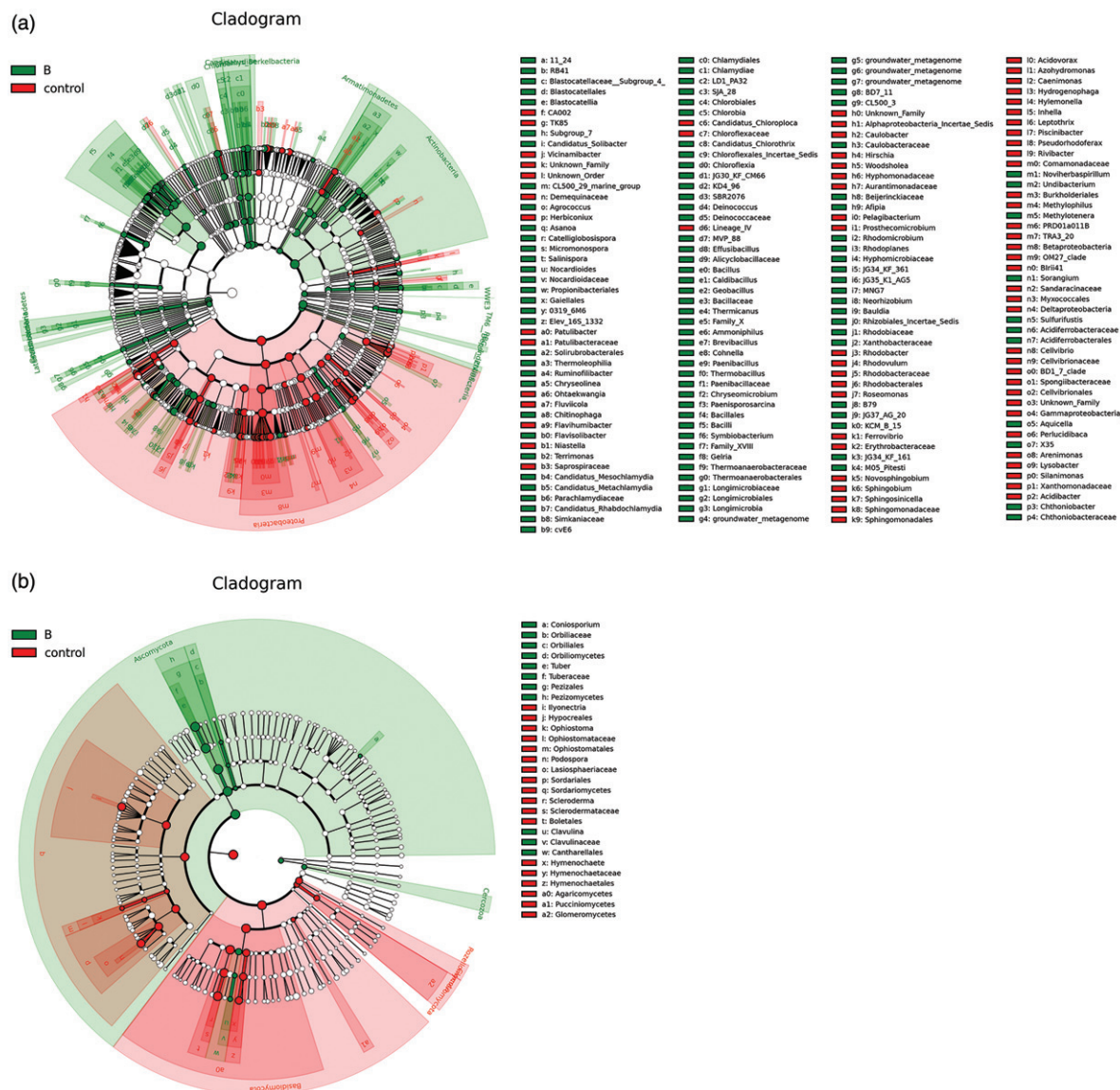
A total of 38 bacterial phyla were identified from the 10 samples (Figure 3(a)). Proteobacteria (44.63%–69.83%), Actinobacteria (1.30%–11.30%) and Firmicutes (1.27%–16.68%) were dominant in all of the samples. The relative abundance of Proteobacteria was significantly lower in treatment



**Figure 2.** Visualization of annotated phylogeny and taxonomy of bacterial (a) and fungal (b) communities associated with *C. avellana* roots with or without *T. borchii* partner based on GraPhlAn analysis. The node sizes correspond to the average relative abundances of the corresponding taxa, arranged in order from phylum to genus. Shadow color on the letter is consistent with the corresponding node color.



**Figure 3.** Taxonomic composition of bacterial and fungal communities associated with *C. avellana* root tips at the phylum and class levels. B, ectomycorrhizae from *C. avellana* mycorrhized with *T. borchii*. Cr, roots from cultivated *C. avellana* without *T. borchii* partner. (a) Bacterial phyla; (b) bacterial classes; (c) fungal phyla; (d) fungal classes. All of the experiments were conducted with five replicates.



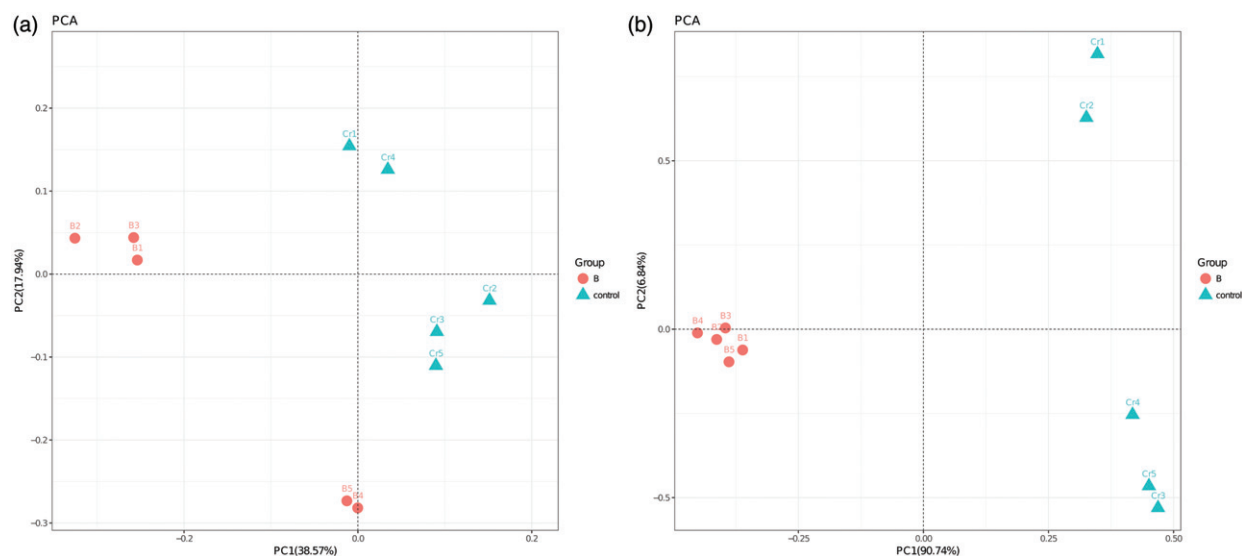
**Figure 4.** Bacterial (a) and fungal (b) taxa with significantly different abundances among communities associated with *C. avellana* roots with or without *T. borchii* partner based on LEfSe analysis ( $p < 0.05$ ). B, ectomycorrhizae from *C. avellana* mycorrhized with *T. borchii*. Control, roots from cultivated *C. avellana* without *T. borchii* partner.

**Table 3.** The 10 most abundant bacterial and fungal genera in *C. avellana* roots with or without *T. borchii* symbionts.

Sample	Bacteria		Fungi	
	Cr	B	Cr	B
Genera	<i>Anaerolineaceae_uncultured</i> (5.78)	<i>Rhizobium</i> (7.93)	<i>Scleroderma</i> (61.63)	<i>Tuber</i> (82.97)
	<i>Comamonadaceae_uncultured</i> (5.46)	<i>Anaerolineaceae_uncultured</i> (4.50)	<i>Ilyonectria</i> (14.48)	<i>Scleroderma</i> (13.12)
	<i>Rhizobium</i> (5.31)	<i>Pedomicrobium</i> (3.80)	<i>Podospira</i> (13.04)	<i>Trechispora</i> (0.82)
	<i>Sandaracinaceae_uncultured</i> (3.01)	<i>Ilumatobacter</i> (3.56)	<i>Hymenochaete</i> (2.81)	<i>Ceratobasidiaceae_unidentified</i> (0.42)
	<i>Woodsholea</i> (2.96)	<i>Streptomyces</i> (3.32)	<i>Ascomycota_unidentified</i> (1.53)	<i>Ascomycota_unidentified</i> (0.28)
	<i>Streptomyces</i> (2.72)	<i>Geobacillus</i> (2.77)	<i>Pyronemataceae_unidentified</i> (1.32)	<i>Phaeoacremonium</i> (0.26)
	<i>Vicia faba</i> (2.46)	<i>Caulobacteraceae_uncultured</i> (2.75)	<i>Rozellomycota_unidentified</i> (1.17)	<i>Monographella</i> (0.23)
	<i>Actinoplanes</i> (2.34)	<i>TM6(Dependentiae)_uncultured bacterium</i> (2.69)	<i>Monographella</i> (0.67)	<i>Trichocomaceae_unidentified</i> (0.22)
	<i>Hydrogenophaga</i> (2.23)	<i>Xanthomonadales_Incertae_Sedis_uncultured</i> (2.12)	<i>Penicillium</i> (0.43)	<i>Humicola</i> (0.21)
	<i>Pedomicrobium</i> (2.14)	<i>Elev-16S-1332_uncultured bacterium</i> (2.11)	<i>Phaeoacremonium</i> (0.26)	<i>Auriculariales_unidentified</i> (0.18)

Average relative abundances are given in brackets (%). Each value is the mean of five biological replicates. B, ectomycorrhizae from *C. avellana* mycorrhized with *T. borchii*. Cr, roots from cultivated *C. avellana* without *T. borchii* partner.





**Figure 5.** Principal Component Analysis (PCoA) of differences among bacterial (a) and fungal (b) communities associated with *C. avellana* roots with or without *T. borchii* symbionts based on weighted UniFrac distances. B, ectomycorrhizae from *C. avellana* mycorrhized with *T. borchii*. Control, roots from cultivated *C. avellana* without *T. borchii* partner.

B than in the control group ( $p < 0.05$ ). However, Actinobacteria were significantly more abundant in treatment B than in the control group ( $p < 0.05$ ).

There were 100 bacterial classes detected, among which  $\alpha$ -proteobacteria (27.87%–41.05%),  $\beta$ -proteobacteria (1.44%–17.46%), Actinobacteria (4.41%–12.14%), and Gammaproteobacteria (5.32%–12.06%) were dominant (Figure 3(b)). Treatment B showed significantly less  $\beta$ -proteobacteria and Deltaproteobacteria than the control group ( $p < 0.05$ ), but contained more Bacilli ( $p < 0.05$ ) (Figure 4(a)). The relative abundance of  $\alpha$ -proteobacteria, Actinobacteria and Gammaproteobacteria did not differ significantly between the two treatments.

A total of 718 genera were detected in all of the samples, and the 10 most abundant bacterial genera in *C. avellana* roots in the two treatments were shown in Table 3. *Rhizobium* (6.62%), *Streptomyces* (3.02%), *Pedomicrobium* (2.97%), and *Ilumatobacter* (2.65%) were the dominant genera in all of the samples. These genera were more abundant in B treatment. The abundance of *Woodsholea* and *Hydrogenophaga* was significantly higher in the control treatment ( $p < 0.05$ ) while that of *Geobacillus* was significantly higher in B treatment ( $p < 0.05$ ) (Figure 4(a)).

### 3.6. Taxonomic analyses of fungal communities

There were 11 phyla detected in all root samples, and Ascomycota (average 59.19%) and Basidiomycota (39.96%) (Figure 3(c)) were dominant in all of the samples. Treatment B contained significantly more Ascomycota ( $p < 0.05$ ), but

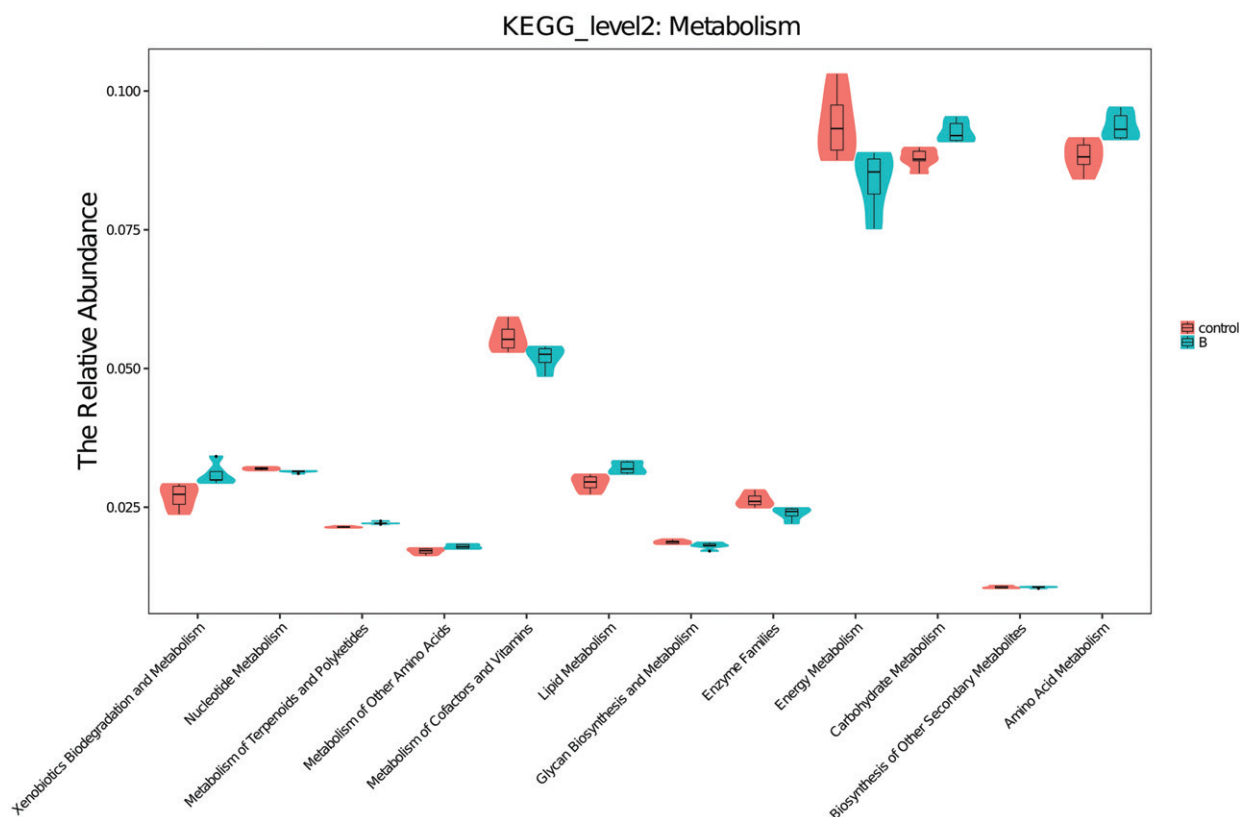
significantly fewer Basidiomycota ( $p < 0.05$ ) compared with the control group.

In terms of the 29 detected classes, Pezizomycetes (42.15%), Agaricomycetes (39.77%), and Sordariomycetes (15.18%) were the most abundant in all of the samples (Figure 3(d)). The abundance of Pezizomycetes was significantly higher in B treatment ( $p < 0.05$ ), while that of Agaricomycetes and Sordariomycetes was significantly higher in the control group ( $p < 0.05$ ) (Figure 4(b)).

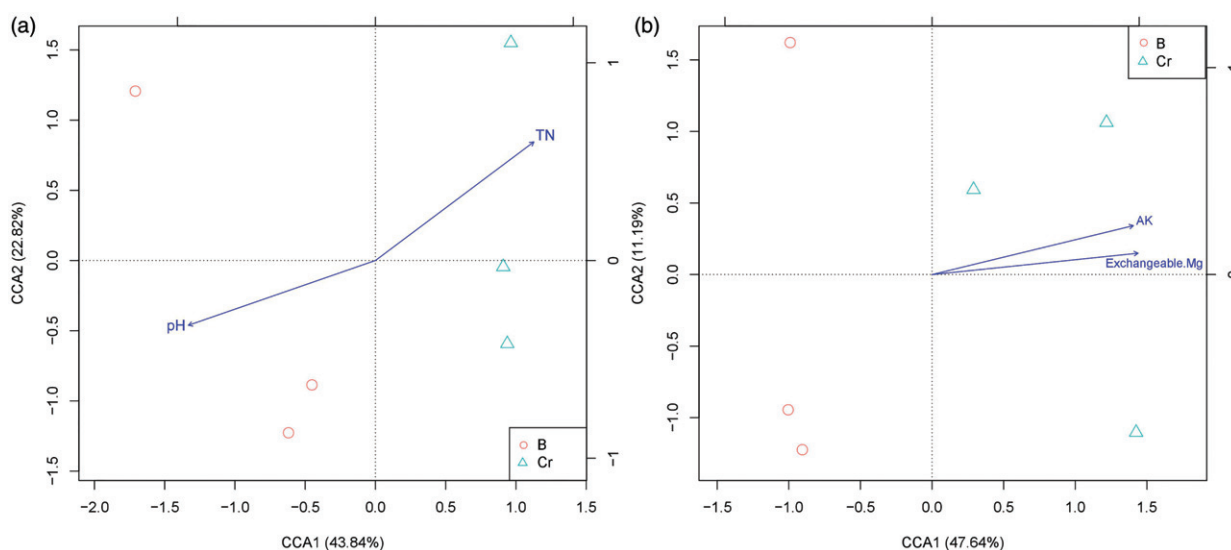
In total, 195 genera were observed, 76 of which were detected in all of the samples, and the ten 10 most abundant fungal genera in *C. avellana* roots in the two treatments were shown in Table 3. *Tuber*, which was the most abundant genus and was only detected in B treatment, accounted for 82.97% of the total in B treatment. The other dominant genera were *Scleroderma* (37.38%), *Ilyonectria* (7.25%), *Podospora* (6.54%), *Hymenochaete* (1.40%) and *Trechispora* (0.51%). *Scleroderma*, *Ilyonectria*, *Podospora* and *Hymenochaete* were all significantly more abundant in the control group ( $p < 0.05$ ) (Figure 4(b)), and almost no *Ilyonectria*, *Podospora* or *Hymenochaete* were observed in B treatment. *Trechispora* was more abundant in B treatment, but this difference was not statistically significant.

### 3.7. Principal component analysis (PCoA) of community differences

Community compositional differences in the bacterial and fungal communities between samples are shown in a PCoA ordination based on the weighted UniFrac distance (Figure 5). Bacterial communities in ectomycorrhizae (Figure 5(a)) were separated from those of the control group at a certain degree,



**Figure 6.** Relative abundances of inferred metabolic functions of bacterial communities associated with *C. avellana* roots with or without *T. borchii* symbionts based on KEGG analysis of PICRUST metabolic predictions. B, ectomycorrhizae from *C. avellana* mycorrhized with *T. borchii*. Control, roots from cultivated *C. avellana* without *T. borchii* partner.



**Figure 7.** Canonical correspondence analysis (CCA) for soil properties and bacterial (a) and fungal (b) communities in *C. avellana* roots with or without *T. borchii* partner. Arrows indicate the direction and magnitude of significant soil variables associated with the microbial community structures. B, ectomycorrhizae from *C. avellana* mycorrhized with *T. borchii*. Cr, roots of cultivated *C. avellana* without *T. borchii* partner. TN: total nitrogen; AK: available potassium; EMg: exchangeable magnesium.

and there were significant differences in the fungal communities between the B and control groups on the first PCoA axis (Figure 5(b)).

### 3.8. Prediction of bacterial metabolism

The metabolic functions predicted from the bacterial taxonomic composition will help us to understand

the ecological potential of the observed bacterial communities. The metabolism of the bacterial was predicted using the PICRUST software based on the KEGG Database (Figure 6). Most of the bacterial flora in the treatment and control groups were predicted to carry out energy metabolism, amino acid metabolism, and carbohydrate metabolism. Metabolism of cofactors and vitamins, xenobiotics



biodegradation and metabolism, and metabolism of terpenoids and polyketides comprised a smaller proportion in the predicted bacterial metabolic profile. Among these pathways, the abundance of xenobiotics biodegradation and metabolism, metabolism of terpenoids and polyketides, lipid metabolism, carbohydrate metabolism and amino acid metabolism was higher in treatment B than the control treatment. However, metabolism of cofactors and vitamins and energy metabolism were more abundant in the control treatment.

### 3.9. Linkage of microbial communities to soil properties

Canonical correspondence analysis (CCA) (Figure 7(a)) showed that total nitrogen and pH significantly affected the bacterial community composition. Total nitrogen was positively correlated with the bacterial communities in Cr, whereas pH was negatively correlated with bacterial communities in Cr.

Fungal communities (Figure 7(b)) were significantly affected by available potassium and exchangeable magnesium. Both soil factors were strongly positively correlated with the fungal communities in Cr.

## Discussion

During the lifecycle of truffles, mycelium and sporocarps gradually released metabolic molecules to sustain their essential survival, but the metabolites were toxic and could inhibit the growth of weeds or other biological colonies, causing an area known as brûlé [37,38]. The unique growth mechanism of truffles may be closely related to the soil properties and the microbial community in ectomycorrhizosphere. In the present study, the results showed that the colonization of *T. borchii* and the synthesis of ectomycorrhizae had a feedback effect on soil properties, which is consistent with the results of previous studies [26,39]. Some parameters were found to be different between truffle soils and control soils. The content of available nitrogen, available phosphorus, available potassium and exchangeable magnesium were all significantly increased compared with controls, indicating that the formation of *T. borchii* ectomycorrhizae promoted the release of effective nutrients of rhizosphere soil, which would help the host plant to absorb mineral elements and grow. Furthermore, canonical correspondence analysis (CCA) reflected that some soil properties, such as total nitrogen and available potassium, were significantly correlated with bacterial and fungal diversity patterns in root tips. Soil properties were also

reported to have obvious effects on bacterial and fungal growth and distribution, and the dynamics of fungal populations seem to be correlated with brûlé formation [26,40].

In terms of the microbial community in root tips during the early symbiosis stage, our results showed that the bacterial richness and diversity were higher in ectomycorrhizae of *T. borchii* with *C. avellana*; however, the mycorrhization of *T. borchii* did not have an obvious influence on the richness and diversity of the fungal community. Other studies have reported that *T. indicum* could shape the microbial communities around it directly or indirectly, and the colonizing populations may come from the surrounding environment, such as the air or water [22], which was similar to the results of the present study. *T. melanosporum* ascocarps could also select some bacteria from the surrounding soil, and this selection may be related to its growth and aroma [21,26,41]. Despite the higher bacterial diversity in ectomycorrhizae compared with the control treatment, some bacterial genera, such as *Woodsholea* and *Hydrogenophaga*, were significantly enriched in the control treatment. In our study, when the truffle mycelia were dominant (82.97% in ectomycorrhizae), the diversity of some endophytic pathogenic fungi, such as *Scleroderma*, *Ilyonectria*, and *Podospora*, were reduced [42]. *Hymenochaete*, a kind of competitive mycorrhizal fungi, also decreased after the colonization of truffle mycelia, which was consistent with the results of a previous study that showed ectomycorrhizal fungi can protect the growth of host plants [43–45]. Furthermore, some wood-inhabiting fungi, such as *Phaeoacremonium* and *Trechispora*, were more abundant in ectomycorrhizae [46].

*T. borchii* mycelia were dominant among the fungal communities in the ectomycorrhizae in our investigation in the early symbiotic stage of truffles. Another study reported that when its ascomata formed, the ectomycorrhizae of *T. magnatum* seemed to be very rare and some other mycorrhizal fungi colonized in the same root tips [44,47,48]. During the mature stage of truffles, its niche may be occupied by other mycorrhizal fungi because of its inability to produce mycelium; therefore, we can speculate that the survival strategies and mechanisms of truffles were different in the different growth periods [47]. The synthesis of ectomycorrhizae is the first step in the artificial cultivation of truffles, and this has an extremely important influence on subsequent work [44,48–50]. Some closely related factors in mycelium synthesis have been found, such as the species of host plants, the surrounding environment, and cultivation management [22]. However, comparison of the microbial

communities associated with truffles and changes in the community composition at different times is difficult [39,51]. Due to high-throughput sequencing technology, we can now analyze the microbial community structure around the ectomycorrhizal fungi and infer the roles of different microbes during the growth of truffles. However, the interactions between these microbial communities and truffles need to be further explored.

## Acknowledgments

We thank LetPub ([www.letpub.com](http://www.letpub.com)) for providing linguistic assistance during the preparation of this manuscript.

## Disclosure of potential conflicts of interest

The authors declare that they have no conflicts of interest.

## Funding

This work was supported by the Science and Technology Support Project in Sichuan Province (2016NYZ0040) and the Sichuan Mushroom Innovation Team.

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